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Susceptibility

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Introduction

African-American women on average present with more advanced breast cancer when compared with Caucasian women. This leads to suboptimal cure rates within this population. Numerous investigators have attempted to explain this discrepancy and determine if it stems from an inherent aggressive biologic behavior or a lack of access to appropriate medical care. It is still controversial, but there is evidence from randomized trials that when controlled for stage, African-American women have similar outcomes compared with their ethnic counterparts. However, it appears that socioeconomic factors lead to delayed screening evaluations and disease that is locally or systemically advanced rather than pre-emptive identification of early stage breast cancer. It would therefore be useful to discover a genetic marker that can serve to identify African-American women who are at increased risk for breast cancer at an age prior to disease development. The *ATM* gene has

been chosen for consideration as a potential marker given its critical role in the maintenance of genomic integrity. Most importantly, we have obtained preliminary data from a pilot study at our institution revealing an increased incidence of *ATM* mutations in African-American women with breast cancer. It was found in this study that 3/7 (43%) African-American breast cancer patients possessed an *ATM* mutation compared with 3/45 (7%) breast cancer patients of all other ethnicities combined ($p=0.026$).

The hypothesis to be tested in this project is that a greater proportion of African-Americans with breast cancer harbor a germline mutation in the *ATM* gene compared to African-American women without breast cancer. An additional objective is to determine the functional impact upon the protein encoded by the *ATM* gene for each mutation identified.

The specific aims of this project are to (1) screen 100 African-American breast cancer patients and 100 African-American women without breast cancer and (2) perform functional studies using cells from patients identified as *ATM* carriers to determine whether each *ATM* mutation identified affects radiosensitivity and levels of the protein encoded by the *ATM* gene for each mutation identified.

To accomplish this work, blood lymphocytes will be isolated from African-American breast cancer patients as well as non-breast cancer controls. DNA is isolated from these cells and each of the coding exons of the *ATM* gene for every patient will be screened for *ATM* mutations using denaturing high performance liquid chromatography (DHPLC). In those exons that display aberrant DHPLC profiles suggestive of a mutation, DNA sequencing will be performed to identify and characterize the mutation. For each person diagnosed as an *ATM* carrier, a lymphoblastoid cell line will be created to analyze *ATM* protein levels.

Body

Progress to obtain DNA samples from subjects was limited for the first year as it was not possible to accrue any subjects into our study since we still do not have final approval from the HSRRB (Human Subjects Review Board) of the DOD for the human subjects protocol and informed consent forms. Although every effort was made to expedite this process during the past year, tentative approval was only received on April 30, 2003, that is contingent upon approval from both the Mount Sinai and NYU IRBs. The process to obtain local IRB approval was only then initiated as I was instructed not to pursue approval from the local IRBs until this material was approved by the HSRRB. At this time, final IRB approval for this project has been given by the Mount Sinai and tentative approval by the NYU IRB with several minor modifications requested.

However, a significant amount of work was accomplished in the past year to establish and optimize the techniques being used in this project for both the genetic screening and functional studies. It should be noted that this work was essentially divided between this project and grant DAMD17-02-1-0503

In terms of the genetic analysis, a high throughput modification for our WAVE DHPLC system, the technique that will be used for genetic analysis of the DNA samples, was instituted during the past year. This upgrade of our system permits a reduction from 10, to

approximately 3 minutes, to accomplish the analysis of each sample, thereby substantially increasing the speed of sample screening. In addition, this modification to the WAVE system appears to also enhance the sensitivity of the DHPLC technique. However, because of this modification, it was necessary to re-optimize all of the temperature and buffer conditions under which the DHPLC is performed for each exon. This work is now complete and we have established the optimal conditions for detection of genetic variants using our high throughput upgraded WAVE system. In addition, we began using the enzyme Optimase, primarily because it has been designed specifically for production of PCR products to be screened using the WAVE system. However, this necessitated defining the optimal PCR temperature conditions for each of the 62 exons using this enzyme.

Work was also initiated to perform the functional studies using wild type and ATM mutant lymphoblastoid cell lines. The first part of this work has been the immunodetection of total p53 and p53 which has been phosphorylated. Since it is known that ATM phosphorylates p53 at ser-15, and given that there are commercially available antibodies that specifically recognize p53 protein that has been phosphorylated at ser-15, it is possible to determine the kinase activity of ATM in various cell types indirectly by determining the percentage of total p53 in a cell that has been phosphorylated by ATM at ser-15.

Briefly, we sediment the cells and then resuspend the cells in lysis buffer (50mM Tris/HCl, 5mM Na₂EDTA, 150 mM NaCl, 0.5% NP40, 1mM DTT, 1mM phenylmethylsulfonyl fluoride [PMSF], 0.5 mM sodium orthovanadate, 1mM sodium fluoride) for 20 minutes on ice. The protein concentration is determined using a modified Bradford assay kit (RC-DC, BioRad). 1mg of cellular protein lysate is incubated with 1 µg of monoclonal anti-p53 (5 µl of a 200 µg/ml stock of 1C12 Mouse Monoclonal #2524, Cell Signaling technology) for 1 hour at 4°C with agitation. Protein-A conjugated to Sepharose beads (Sigma) is added to the mixture and incubated for 1 hr. at 4°C with agitation. The immunocomplex is precipitated by centrifugation, washed with 5 volumes of lysis buffer five times, and boiled in 20 µl Laemmli SDS-PAGE loading buffer (62.5 mM Tris/HCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue, 5% 2-mercaptoethanol) (BioRad). The beads are pelleted again using centrifugation.

The supernatant (containing the protein) is loaded onto a 7.5% precast gel (BioRad) and electrophoresed until the bromophenol blue dye front reaches the end of the gel. The proteins on the gel are transferred to a PVDF membrane (Immuno-Blot, BioRad) in Towbin buffer (25mM Tris/HCl, 192 mM glycine, 20% methanol, BioRad) for 2 hr at 4°C. The membrane is allowed to dry and washed in 25 ml phosphate buffered saline with 0.1% Tween-20 (PBST) twice for 5 min at room temperature (RT). The membrane is blocked with 25 ml PBST buffer with added protein blocker for 1 hr at RT and washed twice with 25 ml PBST for 5 min at RT. To the blocked membrane is added 10 ml of antibody dilution buffer (ADB) containing polyclonal rabbit Phospho-p53 (Ser15) antibody (Cell Signaling technology #9284, diluted 1:2000 [5 µl to 10 ml ADB]) and incubated for 1 hr at 4°C with rocking. The blot is washed twice in 25 ml PBST at RT and incubated for 1 hr at 4°C with rocking in secondary antibody (goat-anti-rabbit conjugated with horseradish peroxidase [GAR-HRP]) diluted to 1:5000 in ADB (2 µl antibody added to 10 ml ADB).

The blot is washed twice in 25 ml PBST at RT. The membrane is incubated in diluted BioRad Amplification Reagent (BAR) for 10 min at RT with rocking, washed four times in PBST with 20% DMSO 5 min at RT, and twice in PBST 5 min at RT. The membrane is

incubated in diluted streptavidin-HRP for 30 min, followed by two washes in PBST 5 min RT. Using the Opti-4CN colorimetric detection from the BioRad kit, 15 ml of the colorimetric solution is prepared and the blot incubated in this solution for about 30 min or until the desired level of sensitivity is attained. The blot is washed in ddH₂O for 15 min. The colorimetrically labeled blot is scanned using a table scanner and each band in the scan quantitated using ImageJ (public domain software downloaded from <http://rsb.info.nih.gov/ij/>). The blot is stripped using 40% acetonitrile, following which it is reprobed using the above conditions, but substituting the polyclonal rabbit Phospho-p53 (Ser15) antibody with polyclonal p53 Antibody (#9282 Cell Signaling Technology). Using numerical values representing the relative amount of each form (whole total p53 protein or Ser-15 phosphorylated p53), a ratio of the two is calculated, determining the percentage of the total p53 protein present in the cell which is phosphorylated at ser-15.

Work was also performed during the past year to establish the assay for immunodetection of ATM protein to determine the level of ATM present in various cell types. To accomplish this, the wild type and ATM mutant cells are precipitated by centrifugation and lysed directly in Laemmli SDS-PAGE sample buffer. The protein level is then quantitated using a Bradford protein assay. For each sample, the Western blot procedure described above is employed, though the antibodies used are as follows: for the first probe, rabbit polyclonal anti-ATM antibody (H-280, sc-15392, Santa Cruz Biotechnology, diluted 1:500 in ADB), and for the second probe, rabbit polyclonal anti- β -Actin (H-196, sc-7210, Santa Cruz Biotechnology, diluted 1:500 in ADB). As β -Actin levels remain constant in cells under a variety of conditions, the quantitation values obtained for the β -Actin probe are used to normalize the ATM values, so an accurate comparison of relative levels of the ATM protein can be made for each cell type.

Key Research Accomplishments

- Establishment of a high through-put enhancement to the WAVE DHPLC system which will be used for this project.
- Optimization of DHPLC buffer and temperature conditions for mutation/polymorphism detection using the high throughput system.
- Optimization of the PCR temperature conditions using Optimase.
- Establishment of the assay for quantitation of p53 phosphorylation on ser-15 by ATM.
- Establishment of the assay for ATM protein level quantitation.

Reportable Outcomes

None

Conclusions

Accrual of patients into this study was limited during the first year of the project due to the lengthy process to obtain approval from the HSRRB for the human subjects protocol and consent forms. However, substantial progress was made to establish and optimize the assays and techniques to be used in this project. We are therefore now in a good position to rapidly accomplish all of the tasks outlined for this project.

References

None

Appendices

None